The EMBO Journal vol.5 no.13 pp.3723–3728, 1986

# Requirements for the formation of plasmid-transducing particles of *Bacillus subtilis* bacteriophage SPP1

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Communicated by T.A.Trautner

**We had previously proposed that the production of concatemeric plasmid DNA in plasmid-transducing SPP1 particles is a consequence of phage-directed rolling-circle-type replication of plasmid DNA. The production of such DNA was greatly enhanced when DNA/DNA homology was provided between phage and plasmid DNAs (facilitation of transduction). Here we present evidence that synthesis of concatemeric plasmid DNA can proceed after phage infection under conditions non-permissive for plasmid replication. We also propose that the naturally occurring homology between plasmid and phage is sufficient to account for the frequency of transduction observed in the absence of facilitating homology. Homology of >47 bp gives the maximal facilitation of plasmid transduction. Recombination is not an essential part in the synthesis of concatemeric plasmid DNA.**

*Key words:* pC194 plasmid/recombination/rolling circle replication/template usage

## Introduction

Plasmid DNA can be encapsulated in the *Bacillus subtilis* bacteriophage SPP1 to produce plasmid-transducing particles. These contain exclusively plasmid DNA, which is organized as a concatemer of unit length molecules with the mol. wt of mature phage DNA (Canosi *et al.*, 1982; Deichelbohrer *et al.*, 1985). In transducing lysates the frequency of plasmid transducing SPP1 particles can be enhanced 100- to 1000-fold when there is homology between the plasmid and the transducing phage DNAs (Deichelbohrer *et al.*, 1985). DNA from any source, providing homology of 500– 3500 bp, will give this type of facilitated transduction. Similar observations had been made by Lofdahl *et al.* (1981) and by Novick *et al.* (1986). In *B. subtilis* the production of lysates with plasmid transducing phage is independent of the known host *rec*-functions (Deichelbohrer *et al.*, 1985).

To account for the observed organization of plasmid-transducing DNA we have proposed that plasmid DNA is replicated by a rolling circle mechanism following phage infection (Figure 1). This type of replication would produce concatemeric plasmid DNA large enough to be recognized for phage packaging. Specifically, we have assumed that replicating phage DNA and plasmid DNA synapse either in regions of natural DNA/DNA homology or within homologous regions provided by genetic engineering of either phage or plasmid. These synapses would represent sites where the replication machinery of the phage may attach to the plasmid template and force plasmid DNA into the production of concatemeric DNA by rolling circle replication.

In this paper we report experiments in which we have analysed three questions relevant to the validity of the proposed model. (i) Is the generation of concatemeric, transducible plasmid DNA, following phage infection, independent of plasmid replication functions? (ii) Can the numbers of transducing particles produced in the absence of provided homology be accounted for by the synapses in regions of natural homology between plasmid and phage DNA? Such experiments were designed to provide also an estimate of the minimal extent of homology required to give facilitated plasmid transduction. (iii) Is genetic flux between plasmid and phage detectable in the region of synaptic pairing?

## Results

### *Production of phage-induced concatemeric plasmid DNA under conditions non-permissive for plasmid replication*

Plasmid pE194 is the only *B. subtilis* plasmid known to be temperature-sensitive for replication (Scheer-Abramowitz *et al.*, 1981; Gryczan *et al.*, 1982). Temperature sensitivity of replication is even more pronounced in the pE194-derived plasmid pE194*ts* (Gryczan *et al.*, 1982), where the *ts* mutation was identified within the gene encoding the initiation function for replication (D.Dubnau, personal communication). Here we have used the chloramphenicol resistance providing derivatives of these plasmids, pBD95 and pBD95*ts* (Gryczan *et al.*, 1982) to study the effect of interference of plasmid replication on transduction. Cells containing either plasmid were shifted from permissive growth at 30°C in the presence of antibiotic to 47°C (pBD95) or 38°C (pBD95*ts*). Growth of cells continued in the absence of antibiotic for three generations, during which time the frequency of plasmid-carrying cells decreased as anticipated from a cessation of plasmid replication (Table I). No plasmid loss occurred when cells were maintained at 30°C. Cells were infected at 0, 1, 2 and 3 generations of growth under these conditions. The lysates obtained were then tested for their ability to transduce the Cm marker of the temperature-sensitive plasmid to *B. subtilis* strain 222 at 30°C. The results obtained (Table I) show that the capacity of cells to produce plasmid-transducing DNA decreased *pari passu* with the decrease of plasmid-carrying cells. Hence transducible concatemeric plasmid DNA can be formed under conditions where the plasmids cannot replicate. Therefore with pBD95 (and also pBD95*ts*, data not shown) formation of concatemeric plasmid DNA is independent of the functioning of the plasmid replication system and depends primarily, if not exclusively, on replication functions provided by SPP1.

### *The relationship between the extent of phage/plasmid DNA homology and the amount of transduction enhancement*

The basic level of transduction of plasmids pC194, pUB110, pE194, pT181 and pLS1 by SPP1 was previously determined (Deichelbohrer *et al.*, 1985; unpublished results) to be of the order of $10^{-5}$–$10^{-4}$ transductants/surviving cell. When homology between SPP1 and plasmid was provided in the preparation of a transducing lysate either by inserting plasmid DNA into the bacteriophage DNA or phage DNA into the plasmid, 100- to 1000-fold enhancement of transduction was observed (Deichelbohrer *et al.*, 1985). This enhanced transduc-

**Table I.** Transduction frequencies at non-permissive temperature

| Temperature of growth (°C) | Time of infection after shift (min) | Percent $Cm^R$ cells | Transductants/ surviving cells |
|---|---|---|---|
| 30 | 0 | 97 | $7.5 \times 10^{-5}$ (1)<br>$4.4 \times 10^{-5}$ (2) |
| 47 | 0 | 96 | $4.2 \times 10^{-5}$ (1)<br>$4.0 \times 10^{-5}$ (2) |
| 47 | 40 | 88 | $4.2 \times 10^{-5}$ (1)<br>$2.5 \times 10^{-5}$ (2) |
| 47 | 90 | 81 | $3.6 \times 10^{-5}$ (1)<br>$3.8 \times 10^{-5}$ (2) |
| 47 | 120 | 50 | $2.1 \times 10^{-5}$ (1)<br>$1.9 \times 10^{-5}$ (2) |

222 cells containing plasmid pBD95 were grown overnight at 30°C in TY + 5 μg/ml Cm. Cells were resuspended into fresh TY + 5 μg/ml Cm and grown at 30°C to late exponential phase. After such growth the culture was divided: one-half was infected with SPP1 and divided into two samples, of which one was kept at 30°C, the other one at 47°C (time 0). The other half was diluted 1/2 into pre-warmed (47°C) TY medium without antibiotic with incubation continuing at 47°C. At times 40, 90, 120 min after the shift, the cells were infected with SPP1 to produce lysates, which were used to transduce 222 cells at 30°C. At each time, the frequency of $Cm^R$ cells was monitored by replica plating from non-selective media. Two separate experiments (1 and 2) were performed. $Cm^R$ cells are the average from the two experiments and are expressed as percentage of cells present at the time of infection.

tion could be attributed to an increase in the number of transducing particles in such lysates. With homologies in the range 500–3500 bp (previously studied), nearly the same transduction enhancement was observed. To determine the relationship between the size of the homologous region and transduction enhancement between the basic level of transduction and the maximal value of transduction observed with a homology of 500 bp, we have studied the enhancement of transduction with homologous regions smaller than 500 bp. The plasmids whose transducibility by SPP1 we analysed (Figure 2), were the pC194/SPP1 chimeric plasmid p1948 and its derivatives. These plasmids confer Cm resistance to cells harbouring them. p1948 originated from inaccurate resolution of pC194 from a SPP1/pC194 chimeric phage genome (Alonso *et al.*, 1986). Plasmid p1948, shown in Figure 2, has a deletion in the pC194 moiety and contains 2500 bp of SPP1 DNA. By using restriction enzyme digestion and by *Bal*31 erosion, whose extent was determined by DNA sequencing, we obtained derivatives of plasmid p1948, which contain the deleted pC194 and fragments of SPP1 DNA ranging from 34 to 2500 bp. SPP1 lysates prepared on cells containing such plasmids were analysed for their capacity to transduce Cm resistance. These values and those of our previous study (Deichelbohrer *et al.*, 1985) are compiled in Figure 3. We find that a plasmid/phage DNA homology of 47 bp is still sufficient to give maximal transduction enhancement, whereas in plasmid p1963 with 34 bp homology this value is markedly reduced.

If we extrapolate the descending part of the curve of Figure 3 towards zero homology, we arrive at a homology value of ~10 bp for the nonfacilitated transduction values of $10^{-5}$–$10^{-4}$. Comparing the DNA sequences of *Eco*RI fragment 1 of SPP1 DNA with those of pC194 and pUB110 or pE194, we realized natural homologies which fall into this range. Identical sequences of 15 and 12 bp were found in the sequenced fragments of SPP1 DNA and in DNAs of pC194 and SPP1 and

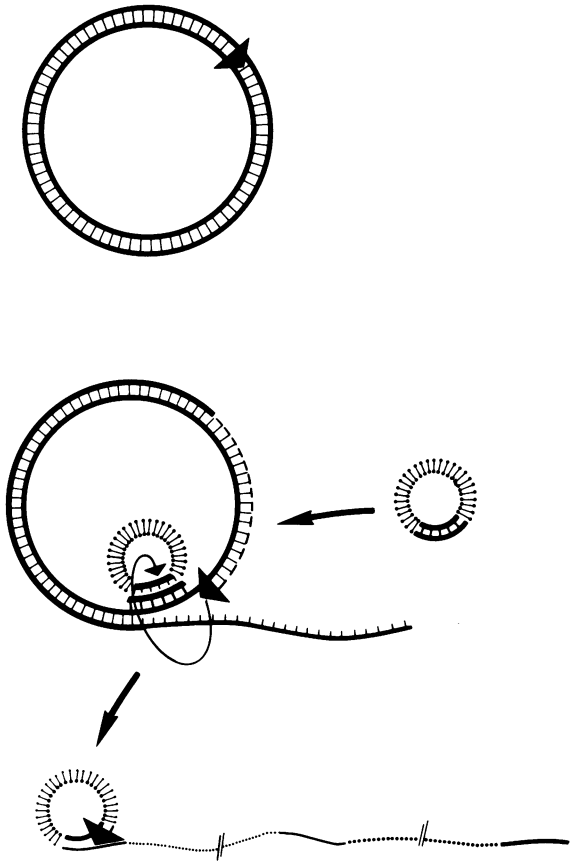

**Fig. 1.** Proposed model for the generation of concatemeric plasmid DNA. The phage chromosome is drawn as a solid double-stranded circle. The triangle represents the phage replicosome. After initiation of clockwise rolling-circle-type replication, phage DNA and plasmid DNA containing phage DNA (solid lines) synapse within the region of homology. Synapsis leads to a template switch of the replicosome from phage to plasmid DNA, initiating rolling-circle-type replication of plasmid DNA. Molecular sizes are not drawn to scale.

pUB110 or pE194, respectively. Statistically other natural homologies would be anticipated between the plasmid and regions of phage DNA, which have not been sequenced. We interpret these results to mean that the differences between basic and facilitated transduction levels are not indicative of different mechanisms but are reflections of the size of homologous regions available for the production of plasmid-transducing DNA.

*Analysis of genetic exchange in facilitated transduction*

We have assumed that the homologous DNA shared by phage and plasmid would generate a synaptic structure with which the phage replication machinery would become associated. This would then lead to a rolling-circle-type replication with the plasmid serving as a template for the phage-specific DNA replication machinery. With this model we expect the majority of unit length plasmids within a concatemer to have the configuration of the plasmid carried by the cells used to prepare the transducing lysate. With our model, conversion within the synaptic region would be the only way through which a genetic flow from phage to plasmid template could occur. On the other hand, recombinant plasmids would be expected if integration of plasmid into phage DNA, e.g. by a Campbell-type event, preceded the plasmid multimers. To test these predictions, we have produced a transducing lysate by infecting cells carrying plasmid pC1941 with SPP1v40. Facilitation of plasmid transduction was not affected by the lack

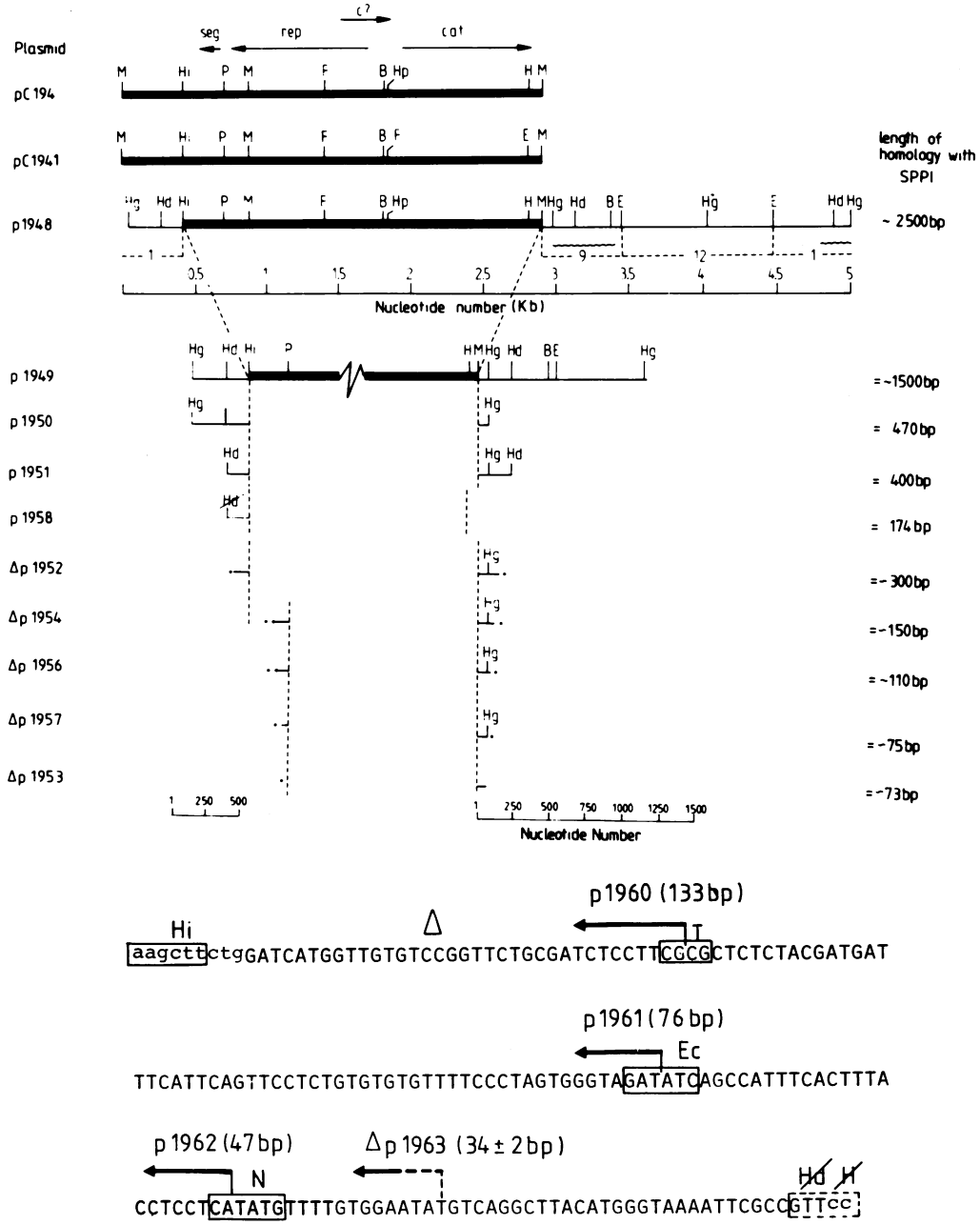


**Fig. 2.** Plasmid pC194 and its derivatives. Heavy lines represent plasmid DNA, light lines SPP1 DNA. The identified genes within pC194 are indicated. In p1948 the SPP1 DNA *Eco*RI fragments 9, 12 and 1 associated with the deleted pC194 are indicated. All DNA had been sequenced except for regions marked by wavy lines. Plasmids p1949, p1950, p1951 were generated by *in vitro* restriction enzyme digestion and religation of DNA containing the deleted pC194. p1958 is a derivative of p1951, in which the small H/Hd fragment had been eliminated. The nucleotide sequence of the SPP1 material (capital letters) of this plasmid extending from the Hi site to the H/Hd join is shown in the lower part of the figure. Deletion derivatives of plasmid p1958 were generated by eliminating from its SPP1 DNA various restriction fragments extending between the Hi site and the T (p1960), Ec (p1961) and N (p1962) sites. Plasmid Δp1963 was produced by digestion of p1958 with N followed by *Bal*31 treatment, which eliminated ~16 bp, followed by Hi digestion and flush end religation. Plasmids Δp1952, Δp1954, Δp1956, Δp1957, Δp1953 represent derivatives of p1951, linearized at its Hd site and subjected to various extents of *Bal*31 erosion. The size of these plasmids was estimated by appropriate restriction enzyme digestion. Letters designate the following restriction sites: B, *Bgl*I; E, *Eco*RI; Ec, *Eco*RV; F, *Fnu*4HI; H, *Hae*III; Hd, *Hin*dII; Hg, *Hga*I; Hi, *Hin*dIII; Hp, *Hpa*II; M, *Mbo*I; N, *Nde*I; P, *Pvu*II; T, *Tha*I.

of complete homology between plasmid and phage. Total DNA obtained from the transducing lysate was degraded by *Kpn*I and *Sma*I. The digest — when analysed by gel electrophoresis — showed the *Kpn*I/*Sma*I restriction pattern of SPP1v40 DNA and a band of non-digestible concatemeric plasmid DNA. No DNA which would hybridize with a $^{32}$P-labelled probe of pC194 DNA was detectable between the bands of plasmid and degraded phage DNA (data not shown; see also Alonso *et al.*, 1986). Plasmid DNA was isolated and part of it was degraded with *Eco*RI or *Hpa*II. The intact and degraded DNAs were used to transform 222 cells to Cm$^R$ and were also analysed by Southern blot analysis with $^{32}$P-labelled pC194 DNA as a hybridization probe. The results of the transformation experiment (Table II) indicate that the transducing DNA is completely refractory to degradation by *Eco*RI. This is paralleled by the conversion of all multimeric DNA to the non-transforming monomeric linear form (Figure 4). *Hpa*II digestion reduces the level of transforming activity of transducing DNA to 75%. This result is compatible with the

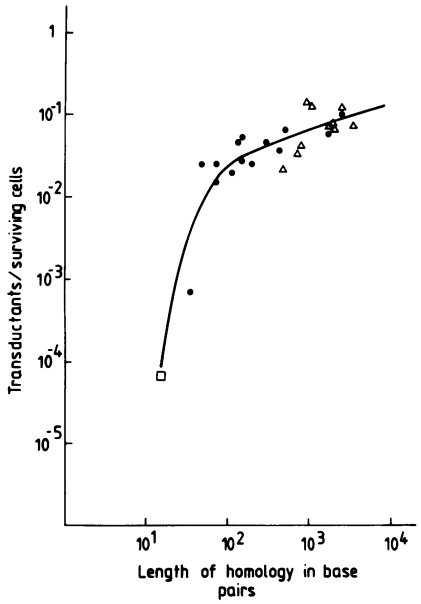


**Fig. 3.** Relationship between frequency of facilitated transduction and DNA homology between plasmid and phage. Triangles (△) are data taken from Deichelbohrer *et al.* (1985). Closed circles (●) represent transduction values obtained with SPP1 and the derivatives of p1948 shown in Figure 2. Their homology values extend between 34 ± 2 bp (Δp1963) and ~2500 bp (p1948). The square (□) represents the frequency of non-facilitated transduction obtained with pC194.

agarose gel analysis, where the amount of concatemeric DNA is reduced in comparison with the non-degraded material, resulting in the appearance of a faint band at the position of linearized monomeric plasmid DNA. No bands with a mobility intermediate between multimers and monomers are detected.

In summary, these results indicate that extensive recombination between pC194 of SPP1v40 and pC1941 has not taken place. There is, however, some transfer of genetic information from the infecting chimeric phage to the concatemer, which is more extensive for the region of the *Hpa*II site than for material corresponding to the *Eco*RI site of pC1941.

## Discussion

The results presented here support our proposed model (Deichelbohrer *et al.*, 1985), describing the generation of concatemeric plasmid DNA subsequent to SPP1 infection through a template switch (Figure 1). (i) Synthesis of concatemeric plasmid DNA was observed after phage infection under conditions where plasmid replication could not occur (Table I). (ii) The relationship in homology-facilitated transduction between transduction enhancement and the size of the DNA homology shared by plasmid and phage suggested that the transduction frequency observed in the absence of provided homology could be accounted for by the presence of natural sequence homology between SPP1 DNA and the plasmids used (Figure 3). (iii) Under conditions of facilitated transduction, recombination between infecting phage and resident plasmid, leading to joint phage/plasmid molecules was not obligatory in the generation of concatemeric, transducible plasmid DNA (Table II, Figure 4).

In spite of these results, details of the mechanism through which the presence of homology between the two replicons leads to the generation of concatemeric plasmid DNA remain unresolved. From the results presented it is plausible that a phage replicosome and not a plasmid replication function is responsible for synthesis

**Table II.** Effect of restriction enzyme digestion on plasmid DNAs[a]

| Enzyme used | pC194 | pC1941 | Transducing concatemeric |
|---|---|---|---|
| None | 175 | 262 | 2710 |
| *Hpa*II | 1 | 262 | 2060 |
| *Eco*RI | 135 | 0 | 0 |

[a]The numbers describe the frequency of $Cm^R$ transformants obtained per 0.15 μg of DNA without or after complete digestion with *Hpa*II or *Eco*RI.

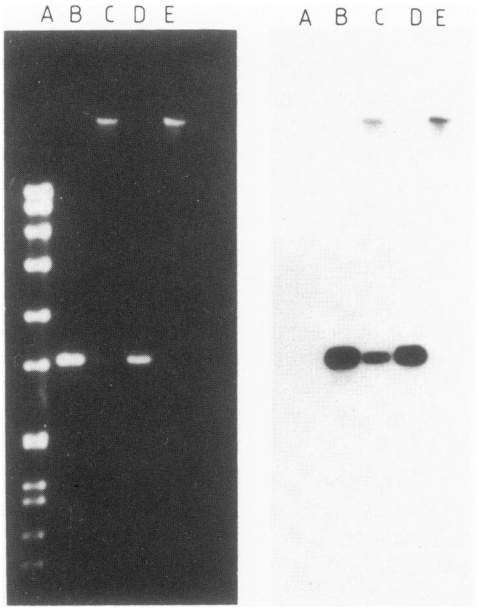


**Fig. 4.** Gel electrophoresis and Southern blot of concatemeric transducing DNA obtained in infections of 222 (pC1941) with SPP1v40 DNA. **A**, SPP1 DNA $M_r$ reference (Ratcliff *et al.*, 1979); **B**, pC1941 DNA, linearized. **C–E**, transducing DNA digested with *Hpa*II, *Eco*RI and undigested, respectively.

of concatemeric plasmid DNA. This result is different from observations of Novick *et al.* (1986), who demonstrated in *Staphylococcus aureus* that the formation of plasmid-transducing particles was reduced under conditions which were non-permissive for plasmid replication. From the independence of concatemeric DNA synthesis from single host-specified *rec* functions and the absence of plasmid-transducing particles containing phage DNA we think that only synapsis between phage and resident plasmid DNAs is a sufficient condition for the formation of concatemeric plasmid DNA. Also this mechanism is different from the one suggested by Novick *et al.* (1986) for staphylococcal plasmid transduction. These authors have proposed that physical recombination between concatemeric plasmid DNA occurring within the homologous region would necessarily precede the formation of chimeric plasmids consisting of multimeric plasmid DNA and phage DNA. Also, P22-facilitated transduction was the consequence of Campbell-type integration of plasmid into the phage (Orbach and Jackson, 1982).

Within the postulated synapsis, genetic flux from phage SPP1-v40 DNA to transducing DNA, which would eliminate the *Eco*RI site of pC1941, was not detectable. In contrast, a small fraction of transducing DNA had obviously become sensitive to degradation by *Hpa*II. This difference of conversion frequency could indicate that synapsis which would encompass the 2-bp sequence

heterogeneity of the *Hpa*II site would be tolerable, whereas the 8-bp difference defining the *Eco*RI site of pC1941 would interfere with synapsis in this region. From the finding that *Hpa*II degradation of the transducing DNA leads exclusively to monomeric plasmid DNA and not to intermediate oligomers, it follows that transducing molecules contain a minor population of concatemers of which each monomer carries the *Hpa*II site of SPP1v40. Hence, gene conversion must have changed that plasmid molecule which serves as a template in rolling circle plasmid replication.

It is an open question whether plasmid/phage interaction occurs at the double-stranded stage as drawn in Figure 1 of both DNA molecules or involves the single-stranded plasmid DNA intermediate identified by te Riele *et al.* (1986). In this context it is important to mention that facilitated transduction of pUB110, which was shown by te Riele *et al.* (1986) to produce very little intermediate single-stranded DNA, occurred with almost the same efficiency as observed for pC194 (Deichelbohrer *et al.*, 1985; and unpublished results). A decisive answer to this question can only come from an analysis of transduction with plasmids whose replication does not involve single-stranded intermediates. Such studies are in progress in our laboratory. Also, we cannot define the molecular events which lead to the postulated template switch within the synapsis region. We could visualize the transition of theta-like to rolling-circle-type plasmid replication in two ways. (i) The synaptic structure could represent a substrate for the initiation of phage-controlled replication on the plasmid template. This would be in analogy to observations made by Luder and Mosig (1984) in T4. (ii) Synapsis would occur between resident plasmid DNA and replicating phage DNA within the replication fork with the consequence of a template switch of the DNA polymerase complex.

Analysing the curve of Figure 3, we were intrigued with the observation from recombination systems analysed in *Escherichia coli* that minimal homology requirements for genetic recombination there fall consistently into the same size range as the homology requirement defined by facilitated transduction in *B. subtilis*. Singer *et al.* (1982) studied recombination between two deletions of T4. The deletions were separated by a region of homology ranging from 40 to 200 bp. Plotting frequency of recombination against the number of homologous base pairs separating the deletions, they realized that the recombination frequency approached zero as the homologous inter-deletion homology approached 50 bp. Similarly, Watt *et al.* (1985), studying recombination in *E. coli* between a plasmid and phage λ derivative, observed a biphasic curve when the recombination frequency was plotted against the length of homologous DNA, within which recombination was forced to take place. Recombination frequency increased 1000-fold over a size range of 20–74 bp, followed by a linear relationship upon further increase of the homologous region. Minimal values for homology requirements of the same order were recently reported by Shen and Huang (1986) and by King and Richardson (1986) also in *E. coli* recombination using an identical approach. The requirement of ~50 bp for maximum efficiency of homology-mediated DNA/DNA interaction in all systems studied, points to a common rate-limiting feature of such interactions irrespective of the host system used.

## Materials and methods

*Bacterial strains, bacteriophages and plasmids*

*B. subtilis* strain 222 (*arg trp*C2) (Trautner *et al.*, 1974) and its plasmid-carrying derivatives were used throughout in all biological experiments. Bacteriophages were SPP1 WT (Riva *et al.*, 1968) and SPP1v40 (Alonso *et al.*, 1986), which is a derivative of SPP1v (Heilmann and Reeve, 1982) into the *Bam*HI site of which *Mbo*I-linearized pC194 had been introduced (Deichelbohrer *et al.*, 1985). Plasmid pC194 was described by Iordanescu (1975). Its sequence has been established by Horinouchi and Weisblum (1982) and Dagert *et al.* (1984). Plasmid pC1941 was constructed and kindly provided by U.Günthert. In this plasmid the unique *Hpa*II was eliminated by filling in the staggered scission, thereby creating two new *Fnu*4HI sites; an *Eco*RI site present on an octomeric oligonucleotide was inserted at the expense of the unique *Hae*III site. Plasmids pBD95 and pBD95*ts* (Gryczan *et al.*, 1982) are derivatives of the thermosensitive replication plasmid pE194 (Scheer-Abramovitz *et al.*, 1981). Plasmid p1948 arose from the SPP1vic/pC194 chimeric phage SPP1v20 through inaccurate resolution (Alonso *et al.*, 1986). The plasmid carries a deletion in its pC194 moiety, and contains 2500 bp of SPP1 DNA derived from the *Eco*RI fragments 9, 12 and 1 (Ratcliff *et al.*, 1979). This plasmid and derivatives of the plasmid containing varying amounts of SPP1 DNA obtained through *in vitro* deletion are shown in Figure 2.

*Transduction and transformation*

Plasmid DNA was prepared according to Canosi *et al.* (1978) or Birnboim and Doly (1979) on preparative or analytical scales. Transducing-phage stocks were prepared as previously described (Deichelbohrer *et al.*, 1985). SPP1 transduction was performed as previously described (Canosi *et al.*, 1982). Transformations were performed according to Rottländer and Trautner (1970). $Cm^R$ transductants or transformants were selected on TY plates containing 5 μg/ml of Cm.

*DNA sequencing*

Relevant fragments of p1948 derivatives (see Figure 2) were cloned into M13mp8 or M13mp9 and sequenced according to the dideoxy method of Sanger *et al.* (1977).

*Other biochemical techniques*

Restriction endonucleases, large fragments of DNA polymerase I and T4 ligase were purchased from Boehringer (Mannheim, FRG). *Bal*31 was from BRL (Bethesda, MD). All enzymes were used as described by the suppliers. Standard techniques were performed as described by Maniatis *et al.* (1982). DNA/DNA hybridizations were performed as described by Southern (1975). DNA probes were labelled with [α-$^{32}$P]dATP according to Rigby *et al.* (1977).

## Acknowledgements

We thank B.Behrens for technical assistance, P.Youngman for providing plasmid pBD95*ts* and D.Ehrlich and R.Novick for communication of unpublished results and stimulating discussions.

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*Received on 25 July 1986; revised on 22 September 1986*

## Note added in proof

In Figure 2 there is an error in the order of *Eco*RI fragment numbers of p1948. The correct order should read 9–1–12–9.